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Discrimination of α-Amino Acids Using Green Tea Flavonoid (−)-Epigallocatechin Gallate as a Chiral Solvating Agent

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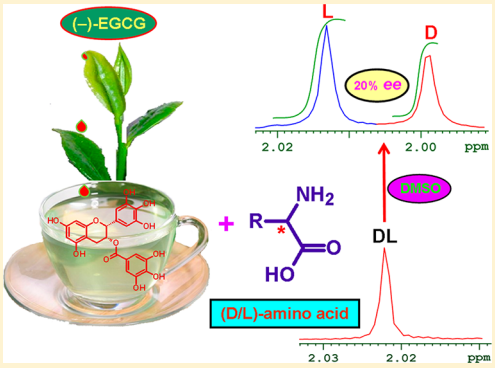
Supporting Information

ABSTRACT: We report a special, hitherto-unexplored property of (−)-epigallocatechin gallate (EGCG) as a chiral solvating agent for enantiodiscrimination of α-amino acids in the polar solvent DMSO. This phenomenon has been investigated by ^{1}H NMR spectroscopy. The mechanism of the interaction property of EGCG with α-amino acids has been understood as arising out of hydrogen-bonded noncovalent interactions, where the −OH groups of two phenyl rings of EGCG play dominant roles. The conversion of the enantiomeric mixture into diastereomers yielded well-resolved peaks for D and L amino acids permitting the precise measurement of enantiomeric composition. Often one encounters complex situations when the spectra are severely overlapped or partially resolved hampering the testing of enantiopurity and the precise measurement of enantiomeric excess (ee). Though higher concentration of EGCG yielded better discrimination, the use of lower concentration being economical, we have exploited an appropriate 2D NMR experiment in overcoming such problems. Thus, in the present study we have successfully demonstrated the utility of the bioflavonoid (−)-EGCG, a natural product as a chiral solvating agent for the discrimination of large number of α-amino acids in a polar solvent DMSO. Another significant advantage of this new chiral sensing agent is that it is a natural product and does not require tedious multistep synthesis unlike many other chiral auxiliaries.



INTRODUCTION

(−)-Epigallocatechin gallate (EGCG), a polyphenolic bioflavonoid abundantly present in green tea,[1,2] is well-known for its biological activities, including anticancer, antidiabetic, antibacterial, and anti-inflammatory, and also as an antioxidant that inhibits cellular oxidation of low density lipo protein in the body. There is also a report on the binding of EGCG to the T-cell receptor CD4 at the gp120 site, establishing it as a potential therapeutic treatment for HIV-1 infection.[3] Because of its numerous health benefits and it is a natural product, EGCG has drawn the attention of many researchers. Though the binding of EGCG to proline-rich proteins like human serum albumin (HAS), pepsin, etc. is well established,[4] there is a paucity of information on its direct interaction with individual amino acids. In this regard, the present study reports the new utility of (−)-EGCG as a chiral solvating agent for discrimination of amino acids and the measurement of their ee in polar solvent DMSO. The determination of ee is of profound importance in drug design, a consequence of the fact that different enantiomers of a chiral drug may have diverse biological properties.[5] For testing the enantiopurity of a chiral compound various analytical techniques are available, such as capillary electrophoresis, crystallization, chiral HPLC, NMR spectroscopy, etc.[6,7] A serious limitation of NMR spectroscopy is that the spectra of enantiopure molecules are identical in the commonly employed achiral solvents, thereby hampering its utility for chiral analysis.

In the classical approach, such problems are circumvented by converting enantiomers to diastereomers by using any of the chiral auxiliaries, such as chiral lanthanide shift reagents, chiral solvating agents, and chiral derivatizing agents.[7] These are specific to functional groups present in the molecules,[8] and the recent book and a review gives the account of the latest developments in the field.[7a,9] A number of studies has been reported on the discrimination of α-amino acids and their derivatives and many solvating agents and derivatizing agents have been reported.[10] The studies have also been reported on β-amino acids.[11] The reported chiral auxiliaries used for the discrimination of α- and β-amino acids are required to be synthesized in the laboratory, which often may involve tedious multiple steps. In this work, we demonstrate the biologically important natural product EGCG as another auxiliary, where a detailed study on the interaction of EGCG with α-amino acids in the DMSO solvent has been carried out. The results clearly provide unambiguous evidence for the utility of EGCG as a chiral solvating agent for enantiodiscrimination of α-amino acids in the polar DMSO solvent.

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RESULTS AND DISCUSSION

The chemical structures of EGCG and α-amino acids used for investigation are reported in Figure 1. The sample preparation and details of experimental and processing parameters are reported in the Supporting Information.[12]

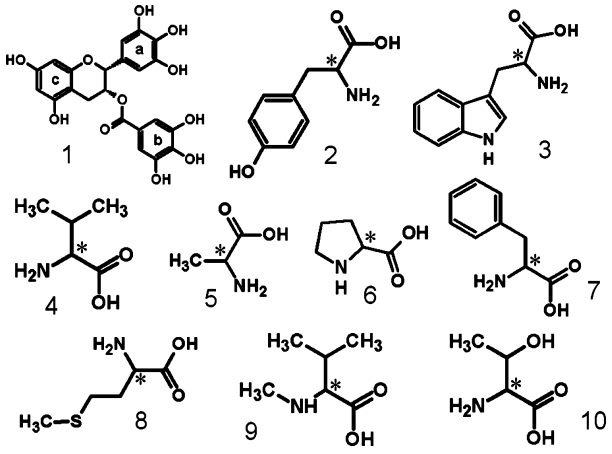

Figure 1. Chemical structures of (−)-EGCG (**1**), tyrosine (**2**), tryptophan (**3**), valine (**4**), alanine (**5**), proline (**6**), phenylalanine (**7**), methionine (**8**), *N*-methylvaline (**9**), and threonine (**10**).

Initially, the ^{1}H NMR spectrum of (D/L)-methionine was studied. The resonating peak pertaining to the SCH_3 group of this enantiopure molecule is reported in Figure 2A, which as expected resulted in a single peak with an indistinguishable

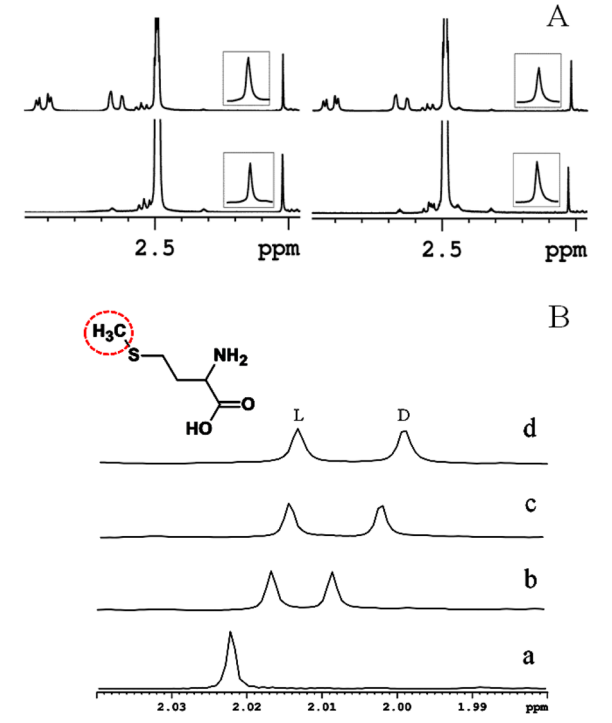

Figure 2. (A) 400 MHz ^{1}H NMR spectra of L- and D-methionine (0.02 and 0.025 mM, respectively); without EGCG (bottom trace) and with EGCG (0.0125 mM) (top trace); the expanded CH_3 peaks are given insets. (B) 400 MHz ^{1}H NMR spectra pertaining to S-CH_3 group of (D/L)-methionine (0.04 mM) at different concentrations of EGCG (*a* = 0, *b* = 0.02, *c* = 0.05, *d* = 0.10 mM, respectively).

superposition of transitions belonging to both the enantiomers. The multistep incremental addition of EGCG to this solution was then carried out to gain insight into their interaction (Figure 2B). At about 0.02 mM of EGCG, the two well-dispersed and identifiable peaks belong to both D and L enantiomers were detected, whose frequency separation enhanced with the increased concentration of EGCG. This observation confirmed that molecule EGCG has the ability to convert enantiomers to diastereomeric complexes. The assignment of peaks to particular enantiomer was made by visual comparison of the discriminated spectrum with the enantiopure spectra, which are given in Figure 2A. It is also evident that higher concentration of EGCG yields better resolution. Such situations are ideal choice when one is interested in high throughput. It may be pointed out that the extent of discrimination, i.e., the frequency separation between the diastereomeric peaks, varies significantly depending on the chiral auxiliary utilized and also the NMR nuclei detected.[10] Further, it also differs significantly for different chemically nonequivalent protons of the given molecule.[10c,d]

Encouraged by the excellent enantiomeric discrimination achieved for (D/L)-methionine, similar experiments were carried out on other amino acids, whose chemical structures are reported in Figure 1, for ascertaining the wide utility of EGCG for chiral analysis. For the molecules utilized for ee measurement the assignment peaks to particular enantiomer was made by comparing the discriminated spectrum with enantiopure spectrum. For brevity, the one-dimensional ^{1}H NMR spectra pertaining to protons $H_{\gamma/\gamma'}$, $H_{\gamma'/\gamma}$, $H_{\beta/\beta'}$, and $H_{\beta'/\beta}$ of (D/L)-proline are reported in Figure 3 for different concentrations of EGCG. However, because of spectral complexity, it is difficult to unambiguously draw conclusion about the discrimination, if any.

Many times, one encounters such complicated situations for diverse reasons, viz., (a) small separation of the discriminated NMR peaks, (b) severe overcrowding of peaks consequent to many protons resonating over a small region, (c) complex multiplet pattern due to many scalar couplings experienced by an interacting spin, and (d) partial resolution of the spectrum with improper baseline correction. Any of these situations severely hinders the identification of peaks and the precise measurement of ee. The prior requirement for chiral analysis in such situations is the simplification of spectral complexity, achieving higher resolution and unraveling of the peaks pertaining to different enantiomers. The use of lower concentration of EGCG being economical, we have employed our reported two-dimensional experimental technique[13] that is selective decoupling in the F_1 dimension. The selective decoupling experiment involves the selective excitation of a proton spin Hi of a molecule by the application of a selective 90° pulse. Couplings between the spin Hi and other proton spins Hj are refocused by the hard 180° pulse during the first evolution period t_1, whereas Hi experiences an overall "360°" rotation due to the application of a selective 180° pulse on Hi. This allows coherence to evolve only according to its chemical shift. The free induction decay is then acquired during t_2. In the resulting 2D spectrum, there is a multiplet structure in the direct dimension due to Hi−Hj interactions but a singlet at each chemical shift in the indirect dimension. Another advantage of this technique is that when there are partially resolved peaks, consequent to the appearance of the different enantiomer peaks in different cross sections, the deconvolution may not always be necessary since areas of the well-isolated

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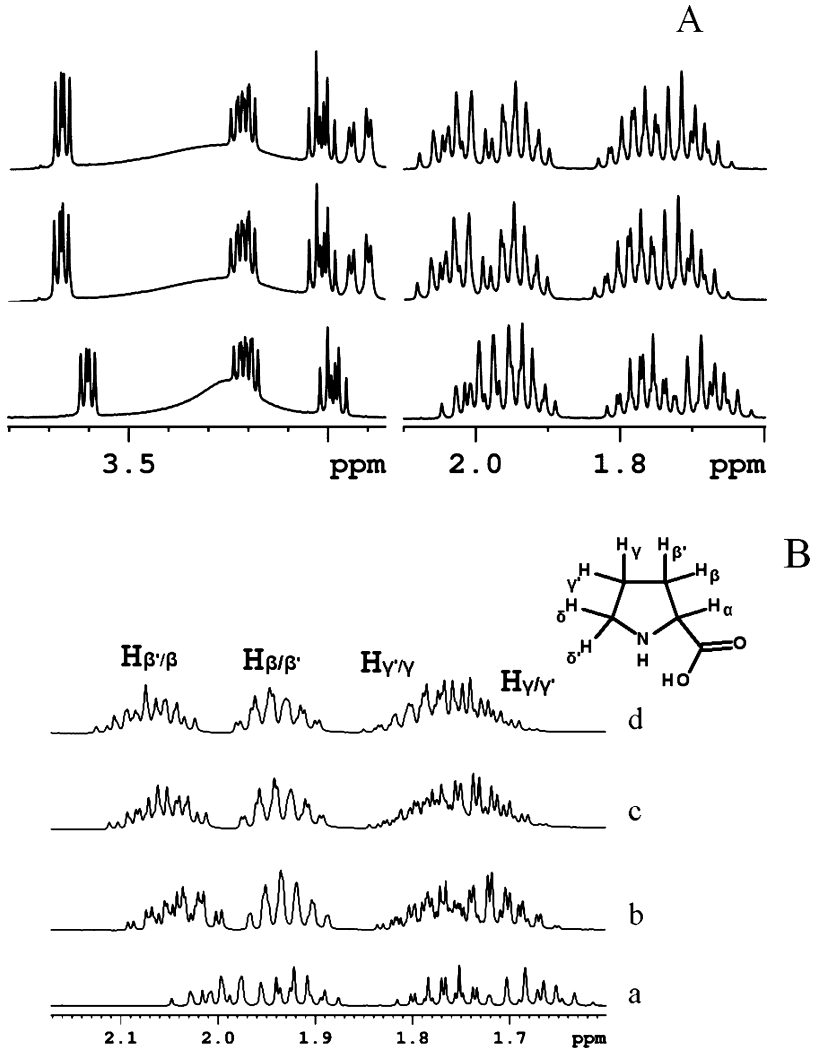

Figure 3. (A) 400 MHz ^{1}H NMR spectra of L-proline (0.029 mM) without EGCG; middle trace: L-proline (0.0273 mM) with EGCG (0.0125 mM); top trace: D-proline (0.0273 mM) with EGCG (0.0125 mM). (B) 400 MHz ^{1}H NMR spectra of (D/L)-proline (0.067 mM) pertaining to protons $H_{\gamma/\gamma'}$, $H_{\gamma'/\gamma}$, $H_{\beta/\beta'}$, and $H_{\beta'/\beta}$ with different concentrations of (−)-EGCG, respectively, from a−d: 0, 0.0296, 0.0654, and 0.1153 mM. Beyond 0.1153 mM of EGCG the spectrum was highly overcrowded and broadened, and hence, EGCG addition was discontinued.

contours yield enantiomeric composition. In the present study, this experimental strategy has been utilized wherever necessary, and representative examples of the selected regions of the two-dimensional F_1 decoupled spectra of (D/L)-tyrosine and (D/L)-proline are given in Figure 4.

It is clearly evident from Figure 4 that there is excellent unraveling of peaks due to different enantiomers. Similar experiments have been carried out on other amino acids reported in Figure 1, and their one- and two-dimensional spectra are reported.[12]

The striking feature of the present study is that the discrimination has been achieved in a polar solvent DMSO. The phenomenon of this weak molecular interaction can be understood by the fact that EGCG is a polyphenol and has many −OH sites available for interaction. Similarly, α-amino acids have $-NH_2$ and $-CO_2H$ functional groups that have more propensities to establish hydrogen bonds. The different possible hydrogen bonds between EGCG and an amino acid are as follows: −NH···OH, −NH···OR, −NH···O═C−, −OH···OH, −OH···OR, −OH···O═C, and −C═O···HO−. If there is a possibility of breaking one of the bonds, the complex will still be retained by the remaining bonds holding them together and the broken hydrogen bond gradually gets reestablished. Thus, the EGCG−amino acid complex attains stability even in a highly polar solvent.

This proposed complex formation is further confirmed by studying an amino acid protected with a −COOH group. When (D/L)-tryptophanmethyl ester hydrochloride was allowed to complex with EGCG, the molecule did not yield any discrimination. Further, when (D/L)-*N*-methylvaline is allowed to complex with EGCG the discrimination could be obtained only for the methyl group covalently bonded to nitrogen. On the other hand, the parent molecule (D/L)-valine yielded discrimination at many chemical sites.[12] This may be attributed to the depleting probability of hydrogen bond formation when compared to the molecule with an unprotected group. We verified this proposition by carrying out a similar experiment with epicatechine which is devoid of the gallate moiety (ring "b", see Figure 1), where it failed to serve as a chiral solvating agent. This observation indicates that the −OH groups of "a" and "b" rings of EGCG or ester group play dominant roles in the formation of hydrogen bonds. The −OH resonances of EGCG also become significantly broadened when the

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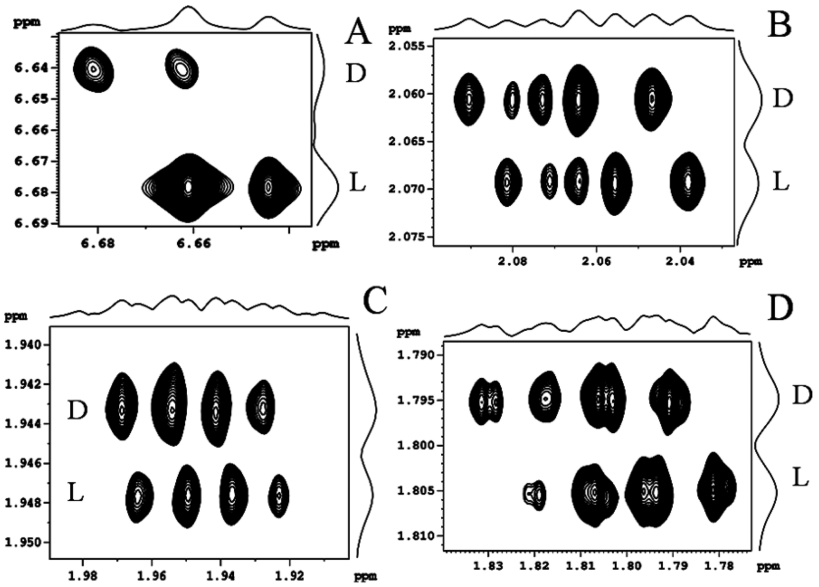

Figure 4. 500 MHz ^{1}H NMR spectra of the selected regions pertaining to: (A) proton H_γ of (D/L)-tyrosine with 0.0178 mM concentration of EGCG; (B-D) peaks pertaining to protons $H_{\beta'/\beta}$, $H_{\beta/\beta'}$ and $H_{\gamma'/\gamma}$ protons of scalemic mixture of (D/L)-proline (the quantities of D and L are 0.0347 and 0.0278 mM, respectively) with 0.0296 mM of EGCG.

discrimination is obtained. This is clearly obvious from the reported spectrum.[12]

With several possibilities of noncovalent interactions of EGCG with amino acids, the question arises whether the peaks observed are enantiodiscriminated or due to multiple structures of the complexes. To answer this question, the studies were carried out with enantiopure L-alanine, and the spectrum of its methyl region was monitored, which gave rise to a doublet because of its coupling with Cα proton, both with and without the presence of EGCG. When this solution was mixed with enantiopure D-alanine, a striking difference was observed in the spectrum and an additional doublet pertaining to D-enantiomer was detected. The spectrum obtained using this protocol, given in Figure 5, unambiguously establishes the fact that EGCG is serving as a resolving agent. This was further exemplified by enhancing the concentration of D-alanine, where there was an increase in the relative intensity of peaks corresponding to D-alanine (between parts C and D in Figure 5A, seen on close inspection). Similar results were obtained with the incremental addition of enantiopure L-alanine to a mixture of D-alanine and EGCG (Figure 5B).

After achieving conclusive evidence for enantiodiscrimination, our subsequent attempt was to explore the applicability of EGCG for the precise measurement of ee. For this purpose, the laboratory-prepared scalemic mixture of 8.2% excess of L-alanine was chosen. The F_1-decoupled 2D spectrum pertaining to the CH_3 group of this molecule is reported in Figure 6. The experimentally measured ee from the ratiometric analysis of areas of contours was 7.7%. Similar experiments were carried out for scalemic mixtures of methionine and proline. The chemical sites of protons utilized to visualize discrimination, and the measured ee are compiled in Table 1.

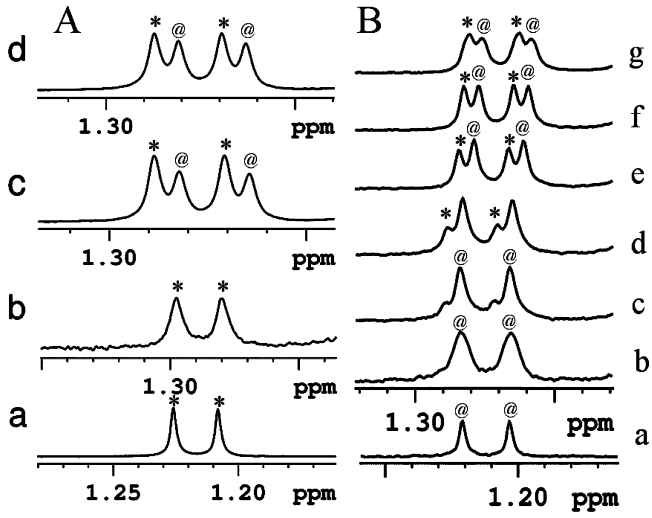

Figure 5. (A) 400 MHz ^{1}H NMR spectra of L-alanine (0.0561 mM): (a) without (−)-EGCG, (b−d) spectra with (−)-EGCG of 0.0156 mM; (b) L-alanine (0.0561 mM); (c) L-alanine (0.0561 mM) and D-alanine (0.0321 mM); (d) L-alanine (0.0561 mM) and D-alanine (0.0481 mM). (B) 400 MHz ^{1}H NMR spectra of D-alanine (0.026 mM): (a) without (−)-EGCG, (b) with EGCG of 0.014 mM, c-g with incremental addition of L-alanine to solution b; (c) 0.004 mM, (d) 0.012 mM, (e) 0.02 mM, (f) 0.026 mM, (g) 0.03 mM. Peaks pertaining to L-isomer marked with * and those pertaining to D-isomer marked with @.

CONCLUSIONS

The present study convincingly established the enantiosensing property of (−)-EGCG and demonstrates its utility for the discrimination of α-amino acids in a polar solvent, DMSO. The significant advantage of this new chiral sensing agent is that it is a natural product and does not require tedious multistep synthesis unlike many other chiral auxiliaries. Most of the chiral auxiliaries lose their chiral sensing ability in polar solvent due to the depletion of hydrogen bonding. However, EGCG can form strong hydrogen bonds where −OH groups of its rings "a" and "b" play dominant roles. The natural abundance of EGCG and its high solubility in DMSO renders it an efficient chiral solvating agent for testing the enantiopurity of α-amino acids. This phenomenon has been demonstrated on several α-amino acids. The 1D ^{1}H NMR spectral analysis is sufficient for the

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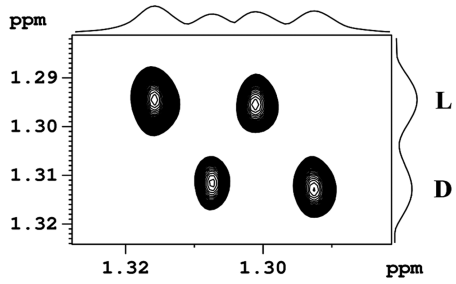

Figure 6. 500 MHz ^{1}H NMR spectrum of CH_3 peak of (D/L)-alanine with an ee of 8.2% of L-enantiomer. The concentration of amino acid is 0.0481 mM and that of EGCG is 0.0249 mM. The experimentally measured ee from the area of the contours is 7.7%.

Table 1. Amino Acids Investigated, Discriminated Peaks for All Amino Acids, and Enantiomeric Excess Calculated from the Experiment for Selected Ones

amino acid	conc of amino acid in DMSO (mM)	conc of EGCG (mM)	% ee from lab-prepared mixture	% ee calcd	resonance exhibited discrimination
alanine[a]	0.0481	0.0249	8.2	7.7	$-CH_3$
methionine[a]	0.044	0.0467	3.2	2.9	$-CH_3$
N-methylvaline	0.049	0.0218	NA	7.3	$-CH_3$
phenylalanine	0.042	0.01558	NA	5	H_2
proline[a]	0.067	0.0296	11.1	11.7	H_4, H_5, H_6[b], H_7
threonine	0.0815	0.036	NA	3.2	$-CH_3$
tryptophan	0.037	0.0158	NA	14.2	H_1, H_3, H_4
tyrosine	0.0315	0.0178	NA	10.9	H_1, H_3, H_4
valine	0.068	0.0358	NA	4.2	$-CH_3$, $-CH_3$

[a]The scalemic mixtures prepared; NA - % ee for these samples were measured experimentally on unknown ratios. [b]The peak used to measure ee.

visualization of enantiomers and also for the determination of ee. Nevertheless, we have utilized a simple and powerful two-dimensional selective F_1 decoupled 2D experiment to achieve discrimination at lower concentration of EGCG. The experiment permitted the unraveling of the severely overlapped peaks and the ratiometric analysis of integral areas of the discriminated peaks yielded the precise enantiomeric contents. Both one- and two-dimensional NMR methodologies facilitated the quantification of optical purity both at higher and lower concentrations of EGCG. We strongly believe that the present study opens up additional avenues for further investigation of biologically important EGCG molecule.

EXPERIMENTAL SECTION

EGCG and all the amino acids were purchased and used without further purification. The NMR spectra were obtained in the solvent DMSO-d_6. The excellent quality of NMR spectra reflects their purity. The concentration of the amino acid and EGCG are reported in the respective legends of the figures in the Supporting Information. All one- and two-dimensional NMR spectra were recorded at ambient temperature (300 K) using 400 and/or 500 MHz NMR spectrometer(s) equipped with BBI and TXI probes, respectively. A temperature control unit was used to maintain the temperature for all the experiments. In situations where there was severe overlap of transitions, the selective F_1-decoupled two-dimensional experiments have been implemented for spectral unraveling. The pulse sequence utilized for 2D experiment and the data acquisition and processing parameters are reported in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Pulse sequence employed, ^{1}H NMR spectra, selective F_1-decoupled 2D spectra, table of acquisition and processing parameters, spectrum of EGCG with and without enantiopure alanine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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